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Nephrogenic diabetes insipidus: An X chromosome-linked dominant inheritance pattern with a vasopressin type 2 receptor gene that is structurally normal

(antidiuretic hormone)

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ABSTRACT **Nephrogenic diabetes insipidus is a rare hereditary disorder, most commonly transmitted in an X chromosome-linked recessive manner and characterized by the lack of renal response to the action of antidiuretic hormone [Arg⁸]vasopressin. The vasopressin type 2 receptor (V_2R) has been suggested to be the gene that causes the disease, and its role in disease pathogenesis is supported by mutations within this gene in affected individuals. Using the PCR, denaturing gradient gel electrophoresis, and direct DNA sequencing, we examined the V_2R gene in four unrelated kindreds. In addition, linkage analysis with chromosome Xq28 markers was done in one large Brazilian kindred with an apparent unusual X chromosome-linked dominant inheritance pattern. In one family, a mutation in codon 280, causing a Tyr → Cys substitution in the sixth transmembrane domain of the receptor, was found. In the other three additional families with nephrogenic diabetes insipidus, the V_2R-coding region was normal in sequence. In one large Brazilian kindred displaying an unusual X chromosome-linked dominant mode of inheritance, the disease-related gene was localized to the same region of the X chromosome as the V_2R, but no mutations were found, thus raising the possibility that this disease is caused by a gene other than V_2R.**

Familial nephrogenic diabetes insipidus (NDI) is a rare disorder characterized by an inability to concentrate the urine in the presence of high endogenous levels of the anti-diuretic hormone [Arg⁸]vasopressin (AVP) (1) or even in response to the exogenous administration of pharmacological doses of AVP analogues (2). Affected males typically present at infancy with polydipsia and hypoosmotic polyuria that may result in dehydration, failure to thrive, mental retardation, or even death. In most families thus far reported, the mode of inheritance follows an X chromosome-linked recessive pattern with variable expression in female carriers (3, 4), although autosomal dominant (5) and autosomal recessive (6) modes of inheritance have also been described. Linkage analyses of families with the X chromosome-linked recessive form of NDI documented tight linkage of the disease-related gene to a number of markers in the subtelomeric region of the long arm of X chromosome (Xq28) (7, 8), the region where the vasopressin type 2 receptor (V_2R) also maps (9, 10). Under normal conditions, AVP (or its analogues) bind to a specific renal tubular receptor, the V_2R that is coupled to adenylyl cyclase via a GTP-binding protein (G protein). Ligand binding results in the activation of adenylyl cyclase and generation of intracellular cAMP (11). Thus, an abnormal, inactivated signal-transduction cascade could explain the renal inability to respond to AVP. Several lines of evidence, in addition to linkage-analysis data, suggested the V_2R gene as the candidate gene in pathogenesis of NDI. The inability to respond to immunologically and biologically normal AVP and the biologically normal GTP-binding protein in patients with NDI are suggestive of a defective receptor (12, 13). Indeed, with the recent cloning of the V_2R (9, 10), mutations within this gene have been found in patients with X chromosome-linked recessive NDI (14–18).

MATERIALS AND METHODS

Laboratory Test Data. Routine laboratory tests (total serum protein, chloride, sodium, potassium, urea nitrogen, creatinine, calcium, phosphorus, urinalysis) were done on seven members of kindred A (Fig. 1) and showed no abnormalities. After informed consent was obtained, these seven family members (III-4, III-8, IV-9, IV-13, V-3, V-4 and V-11, Fig. 1) underwent a short water-deprivation test (Table 1) followed by a s.c. injection of desmopressin acetate (dDAVP; 1 μg Wyeth), a synthetic AVP analogue, the test being done as described (1, 19). Plasma and urine osmolalities were measured with a freezing-point depression osmometer (Advanced Wide-Range Osmometer 3W2, Advanced Instruments, Needham Heights, MA).

DNA Extraction, Southern Blot, and Linkage Analyses. High-molecular-weight DNA was isolated from peripheral blood leukocytes according to a standard protocol (20). DNA samples (5–10 μg) were digested to completion with the restriction enzymes *Taq* I and *Msp* I under conditions recommended by the manufacturer (Boehringer Mannheim) and electrophoretically separated on 0.8% agarose gel; the fragments were then transferred to nylon filters (GeneScreen-*Plus*; DuPont). Prehybridization and hybridization solutions were prepared according to standard protocols (21). The DXS52 probe, a highly polymorphic probe for the distal end of the X chromosome that detects fragments from 6.6 to 0.7 kb with several constant bands (22) was labeled to a specific activity of $\approx 10^9$ cpm per μg by the random-priming method (23) with a commercial kit (Boehringer Mannheim). After overnight hybridization, high-stringency washes were done (0.1× standard saline/citrate; 65°C). The filters were then autoradiographed (Kodak X-AR5 film, after exposure at −70°C for 6–12 h). Another marker (F8C) that lies within 6 centimorgans from the area probed by DXS52 (24) was also used. The F8C dinucleotide-repeat polymorphism was typed exactly as described by Lalloz *et al.* (25).

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Abbreviations: NDI, nephrogenic diabetes insipidus; V_2R, vasopressin type 2 receptor; AVP, [Arg⁸]vasopressin; DGGE, denaturing gradient gel electrophoresis; dDAVP, desmopressin acetate; lod, logarithm of odds.

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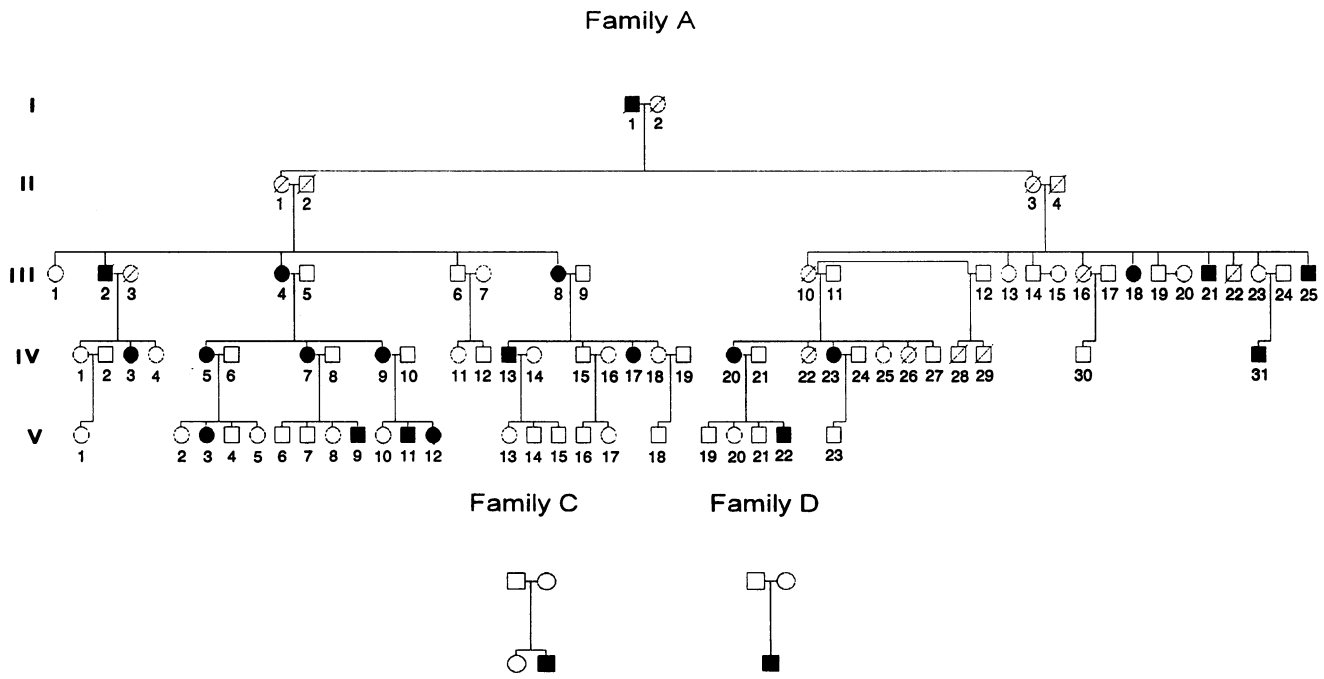

FIG. 1. Pedigree of the kindred A, C, and D. Circles denote females, and squares denote males; a blackened circle or square represents an affected subject, and a slash (/) through the symbol indicates that the subject is deceased. Sequence information in families C and D were obtained from all offsprings and both mothers.

Two-point genetic linkage analysis was done with the computer program MLINK, and multipoint linkage was done with the program LINKMAP, both of the LINKAGE PACKAGE (26).

Mutation Screening. Analysis of the melting properties of the V_2R gene was done with the computer program of Lerman and Silverstein (27). On the basis of this analysis, PCR primers encompassing the entire coding region of the gene, including 120 bases from the 5′ untranslated region, were generated. Whenever possible, primers were chosen from within flanking intronic regions. The second exon is the largest of the V_2R gene and contains 840 bp. Because of its size and specific melting properties, it was subdivided into four different partially overlapping portions so as to enhance the ability to analyze the PCR products on denaturing gradient gel electrophoresis (DGGE). PCR-amplified fragments were analyzed by DGGE in a Hoefer SE620 apparatus as described (28). Samples were electrophoresed between 10 and 13 h at 130 V in a 7.0% acrylamide gel with a parallel gradient of denaturing conditions running from 20% (top) to 90% (bottom) at 60°C. In Table 2, the primer sequences used and the PCR and DGGE conditions are given. For direct sequencing of PCR products, the Sequenase version 2.0 kit (United States Biochemical) was used, employing a biotinylated primer, as described (29).

Table 1. Water-deprivation test in members of an X-linked dominant NDI kindred

	Osmolality, mosm/kg				
	Plasma		Urine		
Patient	Deprivation	dDAVP	Random	Deprivation	dDAVP
V-4 (unaffected)	301	300	990	785	850
III-4	296	295	114	125	125
III-8	310	315	143	155	295
IV-9	300	298	210	225	375
IV-13	294	296	94	97	85
V-3	295	301	135	155	550
V-11	295	315	86	85	95

Deprivation data were obtained at the end of the water-deprivation test. Desmopressin acetate (dDAVP) data were obtained 1 hr after dDAVP administration.

RESULTS

A Different, Apparent X Chromosome-Linked Dominant NDI: Clinical Data and Linkage Analysis. The propositus (proband V-11; Fig. 1) had been referred to one of the authors (L.D.M.) for evaluation of polyuria and polydipsia. Family history was notable for polydipsia affecting several members of this five-generation family. The pedigree of this kindred is depicted in Fig. 1. Individuals I-1, who was a "water drinker," and I-2 both immigrated from Spain in 1899; they were nonconsanguineous, as are all other nonfamily members. Individual IV-31 died at age 25 after a severe dehydration episode, and individuals III-6, IV-15, and IV-23 are affected by anosmia. Individual V-9 suffers from permanent brain damage after an episode of septicemia and prolonged coma. All other members of the family, who were also personally examined by one of us (L.D.M.) (49 in all), have normal intelligence and are generally in good health. Aware of their need for water, they all attend carefully to it. At 3 months of age the propositus had his sole episode of severe dehydration, which was promptly reversed. When examined at age 10, his daily water intake was ≈8 liters, he received no medications, and physical examination was unremarkable. When seen by us, his daily urine output was 12.3 liters with a specific gravity of 1.002. Creatinine clearance was 140 ml/min per 1.73 m^2, and random plasma and urine osmolalities were 295 and 85 mosm/kg, respectively. The results of the water deprivation tests followed by administration of dDAVP in the X-dominant family (kindred A) are shown in Table 1. Urine osmolality of an unaffected family member (V-4) was three times greater than his plasma osmolality at the end of the water restriction, and his urine osmolality rose only 9% in response to dDAVP administration. In all affected patients, urine osmolalities were well below plasma osmolalities before dDAVP administration. In addition, individuals III-4, IV-13, and V-11 showed no further rise of their urine

Table 2. Sequences of synthetic oligonucleotides and optimal conditions for PCR and DGGE

Oligonucleotide	Sequence	Annealing temperature, °C	Dimethyl sulfoxide (10%)	DGGE	V/hr
Exon 1					
3'P	5'-GTC AAA CCC ACT CTG CCC AC-3'	55	+	20–80	130/10
5'P	5'-GCclamp-CA TCT GCC ATG CTG GCA TCT-3'				
Exon 2.1					
3'P	5'-CCA CGG AAG CGG TCG GTG GC-3'	58	+	20–80	130/13
5'P	5'-GCclamp-AC TCC CCC TGC ACA GCA CCC-3'				
Exon 2.2					
3'P	5'-GCclamp-TC ATG GCC AGG ATC ATG TAG-3'	55	+	60–90	130/12
5'P	5'-TTC CAA GTG CTG CCC CAG CT-3'				
Exon 2.3					
3'P	5'-TGC GAC GGC CCC AGG GCT CC-3'	55	+	20–80	130/13
5'P	5'-GCclamp-TC TGC AGA TGG TGG GCA TGT-3'				
Exon 2.4					
3'P	5'-CCC GTC AGC CCT AGC CAC GG-3'	60	–	20–80	130/13
5'P	5'-GC clamp-CG GGG TCA CTG ACT GCT GGG-3'				
Exon 3					
3'P	5'-CCT CTA GAG GCA AGA CAC CC-3'	55	–	40–90	130/10
5'P	5'-GCclamp-CA TCC TGA ACC CAA CCT AG-3'				

GC clamp sequence was 5'-GCG GCC GCC CCT CCC GCC GCC CCC GCC CCG CGG CCG C-3'.

osmolalities after administration of the vasopressin analog, thus confirming the diagnosis of NDI. Individuals III-8, V-3, and IV-9 had low urine osmolality after the deprivation test, which rose in response to dDAVP administration, but to levels well below expected values. Thus, these patients displayed a partial urinary concentrating defect. In particular, individual IV-9 could nearly double her urine osmolality, but this increase was not significantly above her concomitant plasma osmolality.

Fig. 1 shows the pedigree, as well as phenotypes of all tested individuals of family A, suggesting an X-linked dominant disease. There is no male-to-male transmission (see offspring of individual IV-4), there are no unaffected obligate male carriers, and the segregation ratio in the male offspring of affected females is close to 1/2. With the assumption of X chromosome-linked inheritance, 10 living females were obligate carriers on the basis of having an affected father or an affected child (III-4, III-8, III-23, IV-3, IV-4, IV-5, IV-7, IV-9, IV-20, and V-13). Seven of these 10 (all except III-23, IV-4, and V-13) were affected with NDI, and a penetrance value of 0.7 was used for females in linkage analysis. Three additional female patients (II-1, II-3, and III-10, Fig. 1) were also obligate carriers by the above definition, but their clinical status could not be ascertained, as they were deceased at the time of evaluation. Calculation of logarithm of odds (lod) scores for linkage between NDI and F8C probe gave a maximum of 2.29 with $\theta = 0.0$ and for NDI vs. DXS52 probe, a maximum of 1.315 ($\theta = 0.0$), assuming a gene frequency of 10^{-4} (Table 3). Varying the gene frequency from 10^{-3} to 10^{-5} caused only trivial differences in the lod score (data not shown). Most female carriers in this kindred were informative for DXS52 or F8C marker but not for both markers. When multipoint linkage analysis among NDI, F8C, and DXS52 was done, a lod score of 3.36 strongly supportive of linkage (30) was obtained, and this score changed very little between F8C and DXS52. To test whether the disease-related gene in this family cosegregates with the V_2R itself, we used a polymorphism detected by DGGE within exon 3 of the V_2R (31). This analysis showed that, in family A, the disease-related gene cosegregates with the V_2R with no meiotic recombinants. However, only two patients (III-4 and IV-9) were informative, thus limiting any firm conclusions from this latter analysis.

Table 3. Lod scores for genetic linkage between NDI and chromosome Xq28 markers in family A

Marker	Recombination 0.001	0.1	0.2	0.3	0.4
F8C	2.290	1.968	1.554	1.059	0.444
DXS52	1.315	1.206	1.011	0.746	0.412

The maximum multipoint lod score was 3.36, coinciding with the location of marker F8C.

Mutation Analysis of the V_2R in NDI. PCR products from most individuals (affected and nonaffected) with the X chromosome-linked dominant NDI, and three additional families with an apparent X chromosome-recessive form of NDI, covering all three exons, were subjected to DGGE. Fig. 2 shows the migration pattern of the fourth portion of exon 2 (exon 2.4) in kindred B. As clearly shown, an altered migration pattern suggestive of a heterozygous sequence difference in exon 2 is present in the mother of an X chromosome-linked recessive kindred (kindred B), and her affected son displays a homozygous migration pattern. In addition, a pattern suggestive of a sequence difference is shown in the analysis of exon 3 of the same gene (Fig. 2). No other DGGE-analyzed fragments displayed migration abnormalities (data not shown).

Direct DNA sequencing was done on the PCR fragments displaying those migration abnormalities (kindred B), as well as on all PCR products from members of the two Swedish families (kindreds C and D; Fig. 1) not showing altered mobility patterns on DGGE and on three affected patients from the large X chromosome-linked dominant Brazilian family (III-4, IV-13, and V-11 in kindred A). For all fragments, all sequences were done on one strand and on two independent PCR products. In addition, in kindred A, the PCR template used was from two separate DNA extractions. This analysis revealed the following: an A → G alteration in the second base of codon 280 changed the normally encoded tyrosine (TAT) to cysteine (TGT) in the PCR product displaying the migration abnormality on DGGE in family B (Fig. 3). This mutation was present in the son and in his mother. In addition, an A → T alteration in the third base in the codon for Leu-309 (CTA → CTG), which is neutral and encodes the same amino acid, was shown (Fig. 3). This alteration was detected in four of nine healthy unrelated individuals and, thus, represents a polymorphism (31). All PCR products sequenced from kindreds C and D and three affected indi-

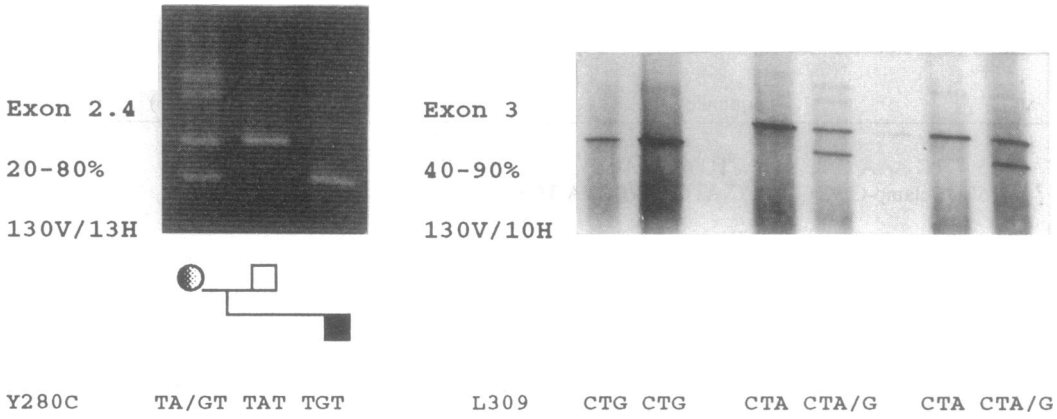

FIG. 2. DGGE analysis of exons 2.4 (*Left*) and 3 (*Right*) in kindred B (X chromosome-linked recessive). Nucleotide sequences from the same patients of codons 280 and 309 are shown at bottom. H, hour.

viduals from kindred A, encompassing the entire coding region of the V_2R, were identical to the published sequences, except the above mentioned polymorphism.

DISCUSSION

NDI is a disorder that must be distinguished from other causes of polyuria. These other causes were clearly ruled out in the large family (kindred A) by routine laboratory tests, the inheritance pattern, and the results of the water-deprivation test (19, 32). In three of the six tested affected members of this family, there was no response to the administration of the AVP analogue, indicating a complete defect of the urinary concentrating mechanism, and in the other three there was an incomplete response, indicating a partial defect. Thus, clinically and biochemically, this family represents a NDI kindred with an unusual X chromosome-linked dominant mode of inheritance. In the three additional small families analyzed, the exact inheritance pattern could not be categorically ascertained, but these patterns are consistent with an X chromosome-linked recessive form, the most common inheritance form in NDI (1). Multipoint linkage analysis data in our large family suggest linkage of the disease with DXS52 and F8C, both markers on the distal end of the X-chromosome (7, 8, 24, 25). Furthermore, data by Bichet *et al.* (34) confirmed the locus homogeneity in 13 independent families with X chromosome-linked recessive NDI.

The mainstay of sex-linked inheritance, either dominant or recessive, is the absence of male-to-male transmission. Because diabetes insipidus is a dominant trait, all female offsprings of an affected male would be expected to display it. Obviously, this outcome was not observed in this family. One possible explanation is partial penetrance. The segregation pattern and linkage data in this kindred indicate that all males carrying the NDI gene are affected, and most female carriers express the disease phenotype as well. The incomplete penetrance in female obligate carriers is most likely related to variable lyonization (X-inactivation) of the normal and mutation-bearing copies of the X chromosome. It seems most likely that the mutation causing NDI in this kindred is unique because females in other X chromosome-linked kindred do not express that disease gene. It is possible that this family has a completely null mutation, and other kindred have mutations that leave some residual activity.

Given the mounting evidence the V_2R plays in the pathogenesis of X chromosome-linked recessive NDI, we analyzed this gene for mutations in all four families. Surprisingly, in only one family, an amino acid alteration (Tyr → Cys) was found. This mutation occurs within the conserved sixth transmembrane domain, and interestingly, the mutant amino acid encoded is cysteine. In five of nine previously reported cases of NDI where amino acid alterations were detected, the mutant amino acid was cysteine (Arg-181, Gly-185, Arg-202, Arg-203, Tyr-205) (14–18). The significance and causes of the predilection for this specific amino acid change are unclear. Recently, Pang *et al.* (33) reported that the Arg-181 → Cys mutation results in a 50–70% reduction of the normal adenylyl cyclase activity in an *in vitro* assay.

In the three other families, no mutations could be demonstrated within the coding region of the V_2R gene. In previously reported cases, in only one case could no mutations within this gene be demonstrated (14). Several possibilities may account for the apparently normal structural V_2R gene product in NDI. (*i*) The possibility that existing mutations

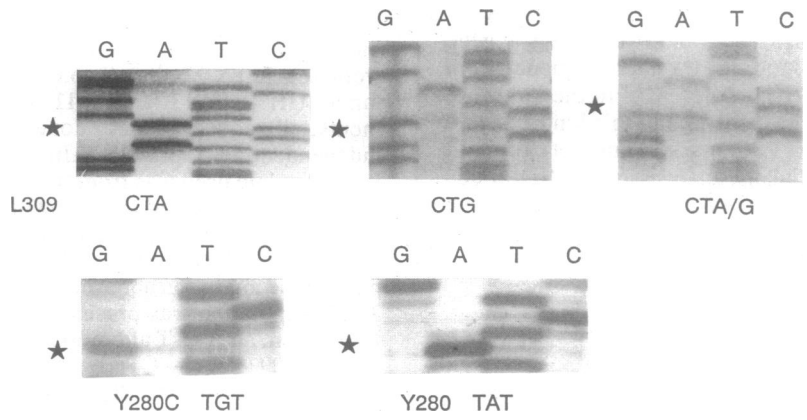

FIG. 3. Direct sequence analysis of the amplified genomic fragments of exon 2.4 (*Lower*) from the affected (*Left*) and unaffected (*Right*) patients and of exon 3 (*Upper*) in kindred B. ★, Polymorphism indicated by sequence beneath each set of blots.

escaped detection must be considered. This explanation seems unlikely because we used a fairly sensitive mutation-detection technique (DGGE) that did detect sequence differences within the V_2R gene, and all fragments were subsequently sequenced on at least two occasions. If a PCR artifact were responsible for eliminating an existing mutation, it is improbable that it would occur twice at exactly the same place, as PCR artifacts occur randomly. (*ii*) Abnormal processing of the initial V_2R mRNA transcript, perhaps as a result of a mutation in an intron, may account for the clinical phenotype. Alternatively, the results could indicate that some posttranslational events (like tissue-specific expression) are responsible for the phenotypically similar disease. Given the limited tissue distribution of the V_2R, ascertaining this possibility may be difficult. (*iii*) The normal V_2R gene product may indicate that an altered regulatory mechanism outside the structural V_2R gene or even another gene, located in proximity to the V_2R and perhaps affecting its expression levels, is involved in NDI pathogenesis.

In summary, another mutation has been described in the V_2R in NDI, further emphasizing its role in disease pathogenesis in some cases. However, in a subset of families, an identical phenotype not displaying gene disruption within the coding region of the V_2R may be associated with a normal structural gene, raising the possibility that altered expression of a normal gene may underlie the pathogenesis of some NDI patients.

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